

Primary-structure requirements for inhibition by the heat-stable inhibitor of the cAMP-dependent protein kinase

(protein phosphorylation/synthetic peptides)

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ABSTRACT The amino acid sequence of the heat-stable inhibitor of the cAMP-dependent protein kinase (PKI) was determined recently [Scott, J. D., Fischer, E. H., Takio, K., Demaille, J. G. & Krebs, E. G. (1985) *Proc. Natl. Acad. Sci. USA* 82, 5732–5736]. An earlier report [Scott, J. D., Fischer, E. H., Demaille, J. G. & Krebs, E. G. (1985) *Proc. Natl. Acad. Sci. USA* 82, 4379–4383] showed that at least part of the inhibitory domain of PKI is located in a 20-residue segment extending from residue 11 to residue 30: Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp-Ile-Leu-Val-Ser-Ser-Ala. In the present study, we further mapped the inhibitory region of PKI by addition or deletion of residues at both ends of this peptide and by substitutions for specific amino acids. The results show that (i) deletion of residues 25–30 did not change inhibitory activity but addition of residues toward the amino terminus increased the inhibitory potency up to 150-fold (K_i 4.8 nM), to a level approaching that of PKI; (ii) replacement of alanine-21 by serine converted the inhibitor into a substrate having a relatively low affinity (K_m 280 μ M) for the enzyme; (iii) replacement of alanine-21 by phosphoserine or α -aminobutyric acid decreased inhibitory activity by a factor of 120 and 20, respectively; (iv) replacement of serine-13 had essentially no effect, whereas substitution of threonine-16 decreased inhibitory activity. The greatest decreases of inhibitory potency occurred with replacements of the arginines in positions 18 and 19.

The heat-stable inhibitor of cAMP-dependent protein kinase (PKI) is a potent inhibitor of the enzyme (1–5). It interacts specifically (2, 3) and competitively (4, 6) with the free catalytic subunit of the kinase (C) formed by dissociation of the holoenzyme (R_2C_2) by cAMP. Rabbit skeletal muscle PKI has been obtained in a homogeneous form by a procedure that employs harsh conditions, including heating at 90°C and acid precipitation (4, 5). Recently, the complete amino acid sequence of the inhibitor from rabbit skeletal muscle was determined by microsequencing techniques (7). PKI contains 75 amino acids ($M_r = 7829$); its amino terminus is blocked. Sequence information confirmed earlier reports (4, 5) that it lacks tryptophan, proline, and sulfur-containing amino acids (7). PKI is a very strong competitive inhibitor of C, exhibiting a $K_i = 0.5$ –2 nM (4, 6). This value is approximately four orders of magnitude lower than the K_m for the synthetic peptide substrate Leu-Ala-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide) commonly used to assay the enzyme (8).

Several investigators have established that basic amino acid residues, in particular arginine side chains preceding the serine or threonine susceptible to phosphorylation, are essential substrate-recognition sites for the cAMP-dependent protein kinase (8–12). A minimal substrate structure of Arg-Arg-Xaa-Ser-Yaa has been proposed (reviewed in ref.

13). The autophosphorylation site in the type II regulatory subunit of the cAMP-dependent protein kinase (RII) contains this structure in its “hinge region” (14–16), and it is thought this segment of RII interacts with C, contributing to inhibition of the latter. The interaction of PKI with C is similar to that of RII, in that arginine residues are essential (4, 6).

In preliminary work (5) it was reported that 2 of the 4 arginine residues in PKI are located in the amino-terminal portion of the molecule, from which an inhibitory peptide could be derived by limited proteolysis (17). The amino acid sequence of PKI (18, 19) has revealed the presence of a pseudosubstrate site, Arg-Arg-Asn-Ala-Ile, that shows similarity to the hinge regions of both types of regulatory subunits, RI and RII (18). Proteolytic cleavage of PKI at arginines destroys its inhibitory activity. The assumption that the pseudosubstrate region is part of the inhibitory site was confirmed by synthesis of a 20-residue peptide (residues 11–30) that is a potent inhibitor with a K_i of 800 nM (7, 18, 19).

In this report, the initial observation that the inhibitory site of PKI is located within a linear sequence covering the pseudosubstrate site has been extended. The inhibitory region has been delineated further by the study of synthetic peptide analogs; the contributions of basic and other specific side chains are examined. A minimal synthetic peptide that might serve as a useful tool for the replacement of PKI as an inhibitor of the cAMP-dependent protein kinase *in vitro* has been defined.

METHODS

Solid-Phase Peptide Synthesis. Peptides were synthesized on a Beckman 990B solid-phase peptide synthesizer (20). More recently, synthesis was performed on an Applied Biosystems Ab430 automatic solid-phase peptide synthesizer as described (21). Cleavage from the resin and deprotection were achieved by incubation in 75% HF/25% anisole for 30 min at 0°C. The amount and composition of each peptide were confirmed by amino acid analysis using the Waters Picotag systems (22). The amino acid sequence of each peptide was determined on a Beckman 890C liquid-phase peptide sequencer as described (23).

Purification of Peptides. Once cleaved from the resin, the peptides were desalted on a 2.5 \times 200 cm column of Sephadex G-10 equilibrated in 1% (vol/vol) acetic acid and then were lyophilized. After being dissolved in a minimal volume, peptides were purified further by reversed-phase HPLC using Vydac semipreparative 1.5 \times 21 cm C_4 columns coupled in tandem, with either an LKB 2150 or a Varian 5000 liquid chromatograph. In each case the solvent system was trifluoroacetic acid/acetonitrile and elution was by increasing acetonitrile concentration. The elution position of individual peptides was a function of their amino acid composition.

Assays. Each peptide was assayed for its ability to inhibit the phosphorylation of the synthetic peptide Leu-Ala-Arg-Arg-Ala-Ser-Leu-Gly (Kemptide) by the cAMP-dependent protein kinase (19).

Calculation of Inhibition Constants. The inhibitory potency of each peptide was assessed by determination of its inhibition constant (K_i). This value was calculated by one of two methods, depending on the inhibitory potency of the individual peptides. A rough indication of the K_i was obtained by determination of the IC_{50} for inhibition (see Eq. 1), using a fixed substrate (Kemptide) concentration equal to its K_m value (taken as 10 μ M).

$$IC_{50} = K_i \left(1 + \frac{[S]}{K_m} \right) \quad [1]$$

When the estimated inhibition constants were greater than 750 nM, K_i values were determined graphically by Lineweaver-Burk plots. For inhibition constants below 750 nM, K_i values were determined by the method of Henderson, as developed for tightly bound inhibitors (24). Each inhibition constant was determined at least five times.

Determination of Michaelis Constants. The K_m of [Ser²¹]PKI-(11-30)-peptide was determined graphically by the method of Lineweaver and Burk.

Proteinase Digestion. Cleavage of [Ala¹³, Glu²⁴]PKI-(11-30) and [Glu²⁴]PKI-(7-30) peptides by *Staphylococcus aureus* V8 protease was carried out for 18 hr as described (19). The resulting fragments were separated by reversed-phase HPLC on two Vydac C₄ columns (1.5 × 21 cm) in tandem, equilibrated in 0.1% trifluoroacetic acid, on an LKB 2150 liquid chromatograph; elution was carried out by increasing concentrations of acetonitrile.

Phosphorylation of Peptides. Peptides were incubated with 300 nM C for 18 hr with ATP. Excess ATP and ADP was removed by ion-exchange chromatography on a column (0.5 × 1 cm) of Dowex AG1X8 anion-exchange resin equilibrated in 30% (vol/vol) acetic acid. The phosphorylated peptide, which did not adhere to the column, was collected in the void volume and was lyophilized.

RESULTS

Substitution of Other Amino Acids for Alanine in the Pseudosubstrate Site of PKI. Utilizing variants of the previously determined (19) inhibitory region of PKI: Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp-Ile-Leu-Val-Ser-Ser-Ala [PKI-(11-30)-peptide], we undertook stud-

ies to determine what effect specific amino acid substitutions and other changes would have on its ability to inhibit C. The first group of peptides studied were those in which alanine-21 of PKI (7) was substituted. As expected, when serine was introduced in place of alanine-21, the resulting peptide, [Ser²¹]PKI-(11-30)-peptide, was a substrate for C and could be phosphorylated. However, it was a relatively poor substrate (K_m 280 μ M). The phosphorylated product, [Ser]^(P)[Ser²¹]PKI-(11-30)-peptide, was appreciably less effective than the parent peptide as an inhibitor of C, exhibiting a 120-fold higher K_i (Table 1). Substitution of α -aminobutyric acid, a threonine analogue, for alanine-21, yielded [Abu²¹]PKI-(11-30)-peptide, which had a 20-fold higher K_i than the parent peptide.

Peptide Chain Length. It was of interest to examine whether extension or shortening of PKI-(11-30)-peptide would affect its inhibitory potency. To facilitate the removal of residues from the carboxyl terminus, a glutamate residue was substituted for aspartate at position 24 to enhance *S. aureus* V8 protease cleavage of the peptide. The peptide synthesized for this purpose, [Ala¹³, Glu²⁴]PKI-(11-30)-peptide also had an alanine residue substituted for serine-13. These changes, at least in sum, had no effect on the K_i for inhibition of C (Table 1). When [Ala¹³, Glu²⁴]PKI-(11-30)-peptide was cleaved, the larger of the two resulting fragments, [Ala¹³, Glu²⁴]PKI-(11-24)-peptide, showed the same affinity for C as the peptide from which it was derived. The smaller fragment, PKI-(25-30)-peptide, was inactive toward C even when tested at concentrations as high as 10 mM. The addition of both fragments, either in equimolar amounts or with a 10-fold molar excess of the smaller peptide, did not affect the inhibition of C by [Ala¹³, Glu²⁴]PKI-(11-24)-peptide (Table 1).

Extending the length of PKI-(11-30)-peptide at the amino terminus resulted in peptides of increased inhibitory potency. Addition of residues 7-10 [PKI-(7-30)-peptide] reduced the inhibition constant by a factor of 3. A similar K_i value was found for [Glu²⁴]PKI-(7-24)-peptide, as expected, since residues 25-30 had been shown to be unimportant. This latter peptide has a glutamate residue substituted for an aspartate to facilitate cleavage by *S. aureus* V8 protease (see above). Further extension toward the amino terminus by inclusion of threonine-5 and -6 decreased the inhibition constant by a factor of 27 (Table 1). Finally, inclusion of residues 1-4 further decreased the inhibition constant. Thus, PKI-(1-24)-peptide exhibited a K_i of 4.8 nM. Surprisingly, the acetylated

Table 1. Inhibition of catalytic subunit of cAMP dependent protein kinase by synthetic peptides related to amino-terminal portion of PKI

Peptide	PKI residues																			K_i , μ M (mean \pm SD)										
	T	D	V	E	T	Y	A	D	F	I	A	S	G	R	T	G	R	R	N		A	I	H	D	I	L	V	S	S	A
PKI-(11-30)										I	A	S	G	R	T	G	R	R	N	A	I	H	D	I	L	V	S	S	A	0.80 \pm 0.04
[Ser ^(P)] ²¹ PKI-(11-30)										I	A	S	G	R	T	G	R	R	N	S ^P	I	H	D	I	L	V	S	S	A	96 \pm 4.5
[Abu ²¹]PKI-(11-30)										I	A	S	G	R	T	G	R	R	N	U	I	H	D	I	L	V	S	S	A	19 \pm 3.3
[Ala ¹³ , Glu ²⁴]PKI-(11-30)										I	A	A	G	R	T	G	R	R	N	A	I	H	E	I	L	V	S	S	A	0.78 \pm 0.10
[Ala ¹³ , Glu ²⁴]PKI-(11-24)										I	A	A	G	R	T	G	R	R	N	A	I	H	E							0.80 \pm 0.20
PKI-(25-30)																								I	L	V	S	S	A	(No inhibition)
PKI-(7-30)					Y	A	D	F	I	A	S	G	R	T	G	R	R	N	A	I	H	D	I	L	V	S	S	A	0.24 \pm 0.07	
[Glu ²⁴]PKI-(7-24)					Y	A	D	F	I	A	S	G	R	T	G	R	R	N	A	I	H	E								0.24 \pm 0.10
PKI-(5-24)				T	T	Y	A	D	F	I	A	S	G	R	T	G	R	R	N	A	I	H	D							0.0088 \pm 0.0020
PKI-(1-24)	T	D	V	E	T	Y	A	D	F	I	A	S	G	R	T	G	R	R	N	A	I	H	D							0.0048 \pm 0.0017
Ac-PKI-(1-24)-NH ₂	^a T	D	V	E	T	Y	A	D	F	I	A	S	G	R	T	G	R	R	N	A	I	H	D	ⁿ						0.035 \pm 0.0080
[Ala ¹³ , ²⁸ , ²⁹ , Abu ¹⁶]PKI-(7-30)					Y	A	D	F	I	A	A	G	R	U	G	R	R	N	A	I	H	D	I	L	V	A	A	A		1.2 \pm 0.5
[Gly ¹⁹]PKI-(7-30)					Y	A	D	F	I	A	S	G	R	T	G	R	G	N	A	I	H	D	I	L	V	S	S	A		120 \pm 7
[Gly ¹⁸]PKI-(7-30)					Y	A	D	F	I	A	S	G	R	T	G	R	N	A	I	H	D	I	L	V	S	S	A			20 \pm 5
[Gly ¹⁴]PKI-(7-30)					Y	A	D	F	I	A	S	G	G	T	G	R	R	N	A	I	H	D	I	L	V	S	S	A		6.6 \pm 1.3
[Gly ²³]PKI-(7-26)					Y	A	D	F	I	A	S	G	R	T	G	R	R	N	A	I	G	D	I	L	V	S	S	A		1.2 \pm 0.3

One-letter amino acid abbreviations are used. S^P, phosphoserine; U, α -aminobutyric acid; ^aT, N-acetylthreonine; ^Dn, aspartic 1-amide.

form of PKI-(1-24)-peptide with an amide group at the carboxyl terminus was a less potent inhibitor than the corresponding peptide with free terminal groups. The decreased affinity could result from partial acetylation of threonyl residues as a consequence of peptide synthesis; side reactions such as these could not be excluded.

Substitutions for Serine, Threonine, and Basic Amino Acid Residues. As noted above, the substitution of an alanine residue for serine-13 apparently had no effect on the ability of PKI-(11-30)-peptide to inhibit C. However, when substitutions were made for all of the serines and the threonine in PKI-(7-30)-peptide, the K_i was increased 5-fold. For this, alanine residues were substituted for serines and α -aminobutyrate was substituted for the threonine to give [Ala^{13,28,29},Abu¹⁶]PKI-(7-30)-peptide (Table 1).

A series of peptides in which each of the arginines in PKI-(7-30)-peptide was replaced by a glycine residue was examined. Replacement of arginine-19 destroyed almost all inhibitory activity, resulting in a 520-fold increase in K_i . Substitution of arginine-18 increased the K_i 90-fold. Substitution of arginine-15 increased the K_i 30-fold. Replacement of histidine-23 by glycine yielded a peptide with a K_i 5-fold higher than that of the parent peptide.

DISCUSSION

The synthesis of peptide inhibitor analogs to PKI indicate that most, if not all, of the structural determinants required for inhibition of the cAMP-dependent protein kinase are located within a linear sequence covering the first 24 residues of the native molecule. These findings extend the original observations of Demaille *et al.* (17), who proposed that the amino-terminal region of the molecule contained the inhibitory site. They isolated an inhibitory peptide ($K_i = 25$ nM) after limited proteolysis and assumed that it originated from the amino terminus because it was refractory to Edman degradation (17). We have previously shown that all inhibitory peptides must contain an intact pseudosubstrate site for the kinase (18, 19) that is structurally similar to the hinge-regions of both types of regulatory subunits (RI and RII) (15, 25) and several substrates of the kinase.

There are structural similarities between substrates and modulators of the protein kinase: they all possess a common site, the sequence Arg-Arg-Xaa-Ala/Ser-Yaa (13). All kinase substrates have a serine or threonine one residue removed from two arginines, whereas both the type I regulatory subunit and PKI have alanine at this position. The minimal effective structure for synthetic peptide substrates is 6-10 residues long, while potent inhibitors contain at least 20-24 residues. This is evidenced by attempts to develop synthetic kinase inhibitors patterned after the heptapeptide substrate Kemptide (26, 27). These were poor inhibitors with inhibition constants 5 orders of magnitude higher than obtained for PKI-(5-24) and -(1-24)-peptides (K_i values of 8.8 and 4.8 nM, respectively). Conversely, the substitution of serine for alanine-21 produced a poor substrate for the kinase, and the phosphorylated derivative [Ser(P)²¹]PKI-(11-20)-peptide was a poor inhibitor ($K_i = 96$ μ M). The high K_m value observed for this peptide probably reflects the presence of additional negative determinants within the structure, such as histidine-23. It has been observed that a positive charge following the serine residue undergoing phosphorylation decreases the affinity for cAMP-dependent protein kinase (27). Replacement of the pseudosubstrate-sequence alanine-21 with α -aminobutyric acid decreased the affinity by a factor of 20. This is not surprising, since introduction of an additional methyl group would make the pseudosubstrate side chain mimic that of threonine, which is often a less preferred substrate for C (11). The kinase has decreased affinity for Kemptide analogs with threonine instead of serine (11).

The detailed biophysical and kinetic studies by several investigators have suggested that substrate recognition by the kinase involves at least two independent steps, both of which can induce conformational changes at the ATP binding site (6, 28-32). One is in response to a subsite of two basic residues, preferably arginines, and the other is due to the hydroxyl group of the target serine. Only the former changes in conformation can occur with inhibitory peptides, since the target hydroxyl group is not present.

Substitution of basic residues, in particular arginine-18 and -19, caused considerable decreases in affinity and may reflect the impaired ability of the positively charged subsite to interact with the kinase. The greatest decrease in affinity was associated with [Gly¹⁹]PKI-(7-30)-peptide, which was 1/520th as potent an inhibitor as the original peptide. This would suggest that arginine-19, the arginine closest to the pseudosubstrate alanine-21, is most important for inhibition.

Replacement of arginine-15 produced a lesser, though still significant, decrease in inhibition. Arginines at this position, 6 residues ahead of the target serine, are important for substrate recognition (12). All protein modulators of cAMP-dependent protein kinase (PKI, RI, and RII) fulfill this criterion (19, 33, 34). Thus, an additional basic site is available for ionic interaction between the modulator proteins and C.

Residues 25-30 are not essential for inhibitory action, since they can be removed without loss of activity. For these experiments, aspartate-24 was replaced by glutamate to provide a better target for *S. aureus* V8 protease (35). These data suggested that most recognition sites for the kinase would be located on the amino-terminal side of alanine-21. With this knowledge, a family of peptides of increasing size and extending toward the amino terminus of the protein were synthesized (Table 1). Peptides beginning at tyrosine-7 displayed a modest increase in affinity (K_i 240 nM); surprisingly, deletion of aspartate-9 produced a more potent inhibitor (K_i 110 nM; data not shown). Aspartate-9 is clearly not essential for inhibition and may even serve as a negative determinant.

PKI is not phosphorylated (19). Nevertheless, a peptide starting at tyrosine-7 was synthesized in which all serine and threonine residues were replaced. The purpose of this experiment was to develop a potent synthetic inhibitor that could not be phosphorylated by any serine or threonine protein kinase. Unfortunately, the affinity of the resulting peptide for C was decreased by a factor of 5. This may be partially explained by the increase in hydrophobicity provided by the substituted amino acids. A more likely hypothesis is that threonine-16 is important for kinase interaction. Such a conclusion can be drawn from the following consideration. Four amino acid substitutions have been introduced in [Ala^{13,28,29},Abu¹⁶]PKI-(7-30)-peptide (Table 1). Residues 25-30 have been shown to be nonessential for inhibition. This excludes a possible negative role for alanines 28 and 29. Likewise, replacement of serine-13 by alanine did not affect inhibition, as seen from [Ala¹³,Glu²⁴]PKI-(11-30)-peptide. Thus, the loss of inhibitory activity can most likely be ascribed to the substitution of α -aminobutyric acid for threonine-16; this replacement causes the loss of a β -hydroxyl group capable of forming a hydrogen bond with the kinase.

The 20-residue PKI-(5-24)-peptide (K_i 8.8 nM) displayed inhibitory properties similar to those of native PKI. The addition of threonines 5 and 6 may have provided another subsite for attachment to the kinase, perhaps through hydrogen-bonding. Addition of residues 1-4 yielded the most potent inhibitor, PKI-(1-24)-peptide ($K_i = 4.8$ nM). The amino terminus of PKI is acylated by a group yet to be determined, though presumed to be an acetyl or a formyl group (7). An acetylated form of PKI-(1-24)-peptide was prepared with an amide group at the carboxyl terminus in an attempt to produce a peptide more closely resembling the

native molecule. The cumulative effect of these two chemical modifications decreased the affinity for the kinase by a factor of 8. There are two possible explanations for these results: (i) PKI is blocked at its amino terminus by another group, or (ii) some modification of serine and threonine side chains may have occurred even though acetylation was performed while the peptide was still bound to the resin and all potentially reactive groups should have been protected (36).

Secondary-structure predictions by the method of Chou and Fasman (37) yielded little indication for an ordered conformation of PKI-(1-24)-peptide. Mildvan and coworkers (38-40) have proposed that bound peptide substrates assume an extended-coil conformation, which minimizes the number of interactions that may occur within the substrate and facilitates enzyme-substrate interaction. They concluded that this might contribute to kinase specificity (40). It is likely that the same conformation is adopted by the pseudosubstrate site of PKI while selected amino acid side chains, located between residues 5 and 16, promote a high-affinity interactive surface with the kinase. Since all inhibitory activity is located in the amino-terminal portion of the molecule, the role of the remaining 50 residues is unclear. The carboxyl-terminal 50 residues might serve a stabilizing function—that is, protect the inhibitory domain from proteolytic degradation. Indeed, it has been observed that the synthetic inhibitory peptides described herein are highly susceptible to enzymatic cleavage and rapidly destroyed *in vivo*; for instance, when injected into *Xenopus* oocytes (M. Cicarelli and J.D.S., unpublished observation).

One of the major objectives in elucidating the amino acid sequence of PKI and locating its inhibitory domain was to determine a minimal structure that would retain strong inhibitory activity toward the kinase. This would then allow the large-scale production of a synthetic inhibitor. On the basis of the information gathered here, we conclude that the 20-residue PKI-(5-24)-peptide could well represent such a minimal structure. Although PKI-(1-24)-peptide is somewhat more potent as an inhibitor, the added complexity of synthesizing the longer peptide is greater than the advantage provided by a slight increase in affinity.

Note Added in Proof. Cheng *et al.* (41) have described the isolation of a highly potent fragment of PKI that would correspond to PKI-(5-25)-peptide in the present paper. A subsequent study (42) reported the inhibitory properties of a series of synthetic peptide analogues patterned after the inhibitory site. Except for some variations in the absolute values of the inhibitory constants, the results of the latter study are reasonably comparable to those reported here.

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